치의학박사 학위논문

The Anti-inflammatory Effect of Human Telomerase-derived Peptide on *Porphyromonas gingivalis* Lipopolysaccharide-induced Cytokine Production

사람 Telomerase 유래 펩타이드가
Porphyromonas gingivalis Lipopolysaccharide에 의해
유도된 사이토카인 발현에 미치는 항염증 효과에 관한 연구

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고 유 진

Abstract

The Anti-Inflammatory Effect of Human Telomerase-derived Peptide on *Porphyromonas gingivalis* Lipopolysaccharide-Induced Cytokine Production

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Objectives. Therapeutic use of the antimicrobial and anti-inflammatory peptides to control microbial infection which is a major cause of pulpal and periapical diseases has attracted considerable interest in endodontic fields. GV1001 peptide is derived from human telomerase reverse transcriptase (hTERT) and was developed as a cancer vaccine. Due to its novel pharmaceutical potential with cell-penetrating ability, as well as anti-

inflammatory activity, GV1001 peptide was suggested as a therapeutic agent to control pulpal inflammation. The purpose of this study was to evaluate the anti-inflammatory effect of GV1001 peptide and its related mechanism in *P. gingivalis* LPS-induced inflammation in human dental pulp cells (hDPCs).

Methods. Dental pulp cells from impacted third molars of human adults were isolated and cultivated. CHO/CD14/TLR2 and CHO/CD14/TLR4 were used to analyze which toll-like receptor (TLR) was stimulated by extracted LPS from *P. gingivalis* (1 μg/ml). *S.* aureus LTA (1 μg/ml) and *E. coli* LPS (100 ng/ml) were used as controls. The ability of intracellular penetration of GV1001 peptide was analyzed by confocal microscopy. The biocompatibility of the peptide (1-50 μM) was measured by MTT assay. Real-time RT-PCR was performed to investigate the expression levels of TNF-α and IL-6 from LPS-stimulated hDPCs. The role of MAP kinase (ERK, p38) signaling pathway on the anti-inflammatory effect of GV1001 peptide was analyzed by western blot analysis. Comparisons between 2 groups were analyzed using Mann-whitney U test (P=0.05).

Results. *P. gingivalis* LPS induced CD25 expression via TLR2. GV1001 peptide was predominantly located in the cytoplasm of hDPCs. The peptide down regulated *P. gingivalis* LPS-induced TNF-α and IL-6 production in hDPCs ($P < 0.05$) without showing significant cytotoxicity. Furthermore, the treatment of GV1001 peptide markedly inhibited the phosphorylation of MAP kinases (ERK and p38) in LPS-stimulated hDPCs.

Conclusions. GV1001 peptide inhibited TNF-α and IL-6 production through reducing *P. gingivalis* LPS-induced phosphorylation of ERK and p38 MAPK in hDPCs, which indicates that the peptide may have anti-inflammatory activity.

Keywords: GV1001 peptide, Human telomerase-derived peptide, *Porphyromonas gingivalis* LPS, MAP kinase (ERK, p38), Anti-inflammatory activity

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Contents

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1. Introduction

Caries, cracks, fractures and leakage from restorations provide pathways for microorganisms and their toxins to enter the pulp. Odontogenic infections are generally caused by polymicrobial and dominated by anaerobic bacteria (1). The response of the pulpal irritation caused by bacteria is inflammation and eventually pulp necrosis may occur. The inflammation can spread to the surrounding alveolar bone and cause periapical

pathosis. In this process, bacterial lipopolysaccharides (LPS) play a potential role in pulpal inflammation. LPS can induce the expression of pro-inflammatory cytokines and chemokines, and elicit the innate immune response in dental pulp cells (DPCs) (2). Recognition of pathogens and subsequent initiation of innate inflammatory/immune events in the dental pulp appear to result from engagement of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed by these cells (3).

Signaling pathways initiated by engagement of TLRs from bacterial components lead to enhanced transcription of genes responsible for the expression of cytokines, chemokines, adhesion molecules, and other mediators of the inflammatory response associated with bacterial infection. Of note, the activation of mitogen-activated protein kinases (MAPKs) is important in the production of inflammatory cytokines by LPS stimulation (4). The MAPK family includes extracellular-signal-related protein kinase (ERK), c-JUN N-terminal kinase/stress-activated protein kinases (JNK/SAP) and p38 MAPK (5). The MAPK signaling pathway is involved in various kinds of cellular processes including differentiation, development, proliferation and survival, as well as cell death, depending on cell type and stimulus (6, 7). MAPK signaling is activated by LPS stimulation during the induction of local pro-inflammatory response (8-10).

Telomeres are specialized structures at the ends of chromosomes that have a role in protecting the chromosome ends from DNA repair and degradation (11). Telomerase is a cellular reverse transcriptase (TERT, telomerase reverse transcriptase) which prevents premature telomere attrition and maintains normal length and function (12). Human

telomerase reverse transcriptase (hTERT) has become an attractive target for cancer vaccines due to it being expressed in 85-90% of human cancer tissues, whereas it is almost never expressed in normal tissues (13). GV1001 peptide, which is a peptide corresponding to amino acids 611-626 of hTERT (EARPALLTSRLRFIPK), has been developed as a vaccine against various cancers and has been reported to have the ability to penetrate into various cells, including cancer cell lines and primary blood cells (14).

GV1001 peptide was found to localize predominantly in the cytoplasm and could successfully deliver macromolecules such as proteins, DNA and siRNA into cells (14). Because of this novel pharmaceutical potential and cell-penetrating ability, as well as its own anti-cancer activity, GV1001 peptide is very promising for use in the medical field.

Recently, the extra-telomeric functions of hTERT have been suggested regarding anti-apoptotic, anti-aging, antioxidant and anti-inflammatory effects (15-17). Due to its novel pharmaceutical potential with cell-penetrating ability, as well as anti-inflammatory activity, GV1001 peptide was suggested as a therapeutic agent to control pulpal inflammation.

The purpose of this study was to evaluate the anti-inflammatory effect of GV1001 peptide and its related mechanism in *P. gingivalis* LPS-induced inflammation in human dental pulp cells (hDPCs).

2. Materials and Methods

Synthesis of GV1001 peptide

All of the peptides used in this study were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl)-based solid-phase method and characterized by Peptron Inc. (Dae-jeon, Korea). The purities of all peptides used in this study were greater than 95%, as determined by high-performance liquid chromatography.

Cultivation of human dental pulp cells

This study was approved by the Seoul National University Dental Hospital Institutional Review Board. The impacted third molars of human adults were collected from 18- to 22-year-old patients without systemic diseases after obtaining informed consent. The isolated dental pulp was cut into small pieces and digested in a solution of 3 mg/mL type I collagenase and 4 mg/mL dispase (Sigma Aldrich, St Louis, MO, USA) for 30–60 min at 37°C. Subsequently, the solution was filtered through a 70-mm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA). The single-cell suspensions were seeded in 35- or 60-mm culture dishes and maintained in a culture media consisting of α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco-BRL., Life Technologies Inc, Gaithersburg, MD, USA), 0.292 mg/mL glutamine (Invitrogen), 100 units/mL penicillin G, 100 mg/mL

streptomycin, and 50 mg/mL ascorbic acid (Sigma Aldrich). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and cells between 3 and 4 passages were used in the following experiments.

Extraction and verification of LPS from *P. gingivalis*

P. gingivalis ATCC 33277 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured with brain heart infusion broth (BHI; BD Bioscience, Sparks, MD, USA) supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) in anaerobic conditions at 37°C. LPS was extracted from *P. gingivalis* (10 μg/ml) cultured according to the method described by Lee *et al*. (18). *E. coli* LPS (O111:B4) was purchased from Invivogen (San Diego, CA, USA). To confirm the purity of extracted LPS, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) was carried out. The LPS was stained with silver nitrate and coomassie blue. *E. coli* LPS was used as a positive control.

Recognized receptors for *P. gingivalis* LPS by CHO/CD14/TLR2 or CHO/CD14/TLR4

CHO/CD14/TLR2 and CHO/CD14/TLR4 were used to evaluate which TLR was used by LPS of *P. gingivalis*, as described previously (19). These cell lines express a gene encoding a membrane CD25 driven by the human E-selectin promoter, which contains NF-κB binding sites. The cells were grown in Ham's F-12 medium (Gibco, Rockville,

MD, USA) supplemented with 10% FBS, 1 mg/mL G418 (Calbiochem, La Jolla, CA, USA) and 400 U/mL hygromycin B (Calbiochem) at 37°C. When the cells were 80% confluent, they were treated with *P. gingivalis* LPS (1 μg/mL), *E. coli* O111:B4 LPS (100 ng/mL) or *Staphylococcus aureus* LTA (1 μg/mL) (InvivoGen) for 16 h in the presence of 10% FBS. The cells were then washed with PBS, detached with 2 mM ethylenediaminetetra-acetic acid (EDTA) in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD25 at 4°C for 30 min. After washing with PBS, CD25 expression in the cells was analyzed by flow cytometry. FITC conjugated mouse immunoglobulin G (IgG) was used as a control for non-specific binding.

Internalization of GV1001 peptide by confocal microscopy

hDPCs were seeded and cultivated in 2-chamber glass slides (Nunc, Roskilde, Denmark) for 12 h. After washing with PBS, cells were incubated in serum-free OPTI-MEM for an hour. FITC-labeled 1, 10 and 50 μM of GV1001 peptides were added to cells and incubated for 2 h. The cells were fixed with 4% paraformaldehyde solution for 20 min at room temperature. The cells were stained with 0.3 μM of DAPI (4,6-diamidino-2-phenylindole, Molecular Probes®) 642⁄661 nm (Invitrogen) to visualize nuclei and were subjected to confocal microscopy. Co-localization of the peptides and nuclei was assessed using an FV1000 lager scanning confocal microscope (Olympus, Tokyo, Japan).

Cytotoxicity assay of GV1001 peptide on hDPCs

To evaluate the cytotoxicity of GV1001 peptide on hDPCs, cells (2×10^5 /well) were treated with several concentrations of GV1001 peptide (0, 1, 5, 10, and 50 μM/well) for 48 h. The cells were incubated with 5.7 mol/L of MTT solution for 4 h in a tissue-culture incubator. A 200 μL quantity of dimethyl sulfoxide solution was then added to the cell-culture wells, and the plates were shaken for 10 min at room temperature to dissolve the precipitated formazan crystals. The solution was centrifuged for 10 min, and the optical density of the supernatant was measured at wavelength of 540 nm using an ELISA plate reader (PowerWave X 340; BioTek Instruments, VT, USA). 0.9% NaCl solution was used as a negative control. The MTT assay was performed three times.

Investigation of MAP kinase (ERK, p38) by western blot analysis

After addition of 0.75 μM or 10 μM GV1001 peptide to LPS-treated cells, the samples were prepared for electrophoresis and were separated using 10% sodium dodecyl sulfate–polyacrylamide gel with a previously established buffer system (20). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and blocked by Tris-buffered saline (TBST, 0.05% Tween-20) containing 5% non-fat dried milk. The membranes were then incubated with anti-p-ERK and anti-p-p38 (Cell Signaling Technology, Beverly, MA, USA) antibodies and subsequently washed with TBST. Antigen–antibody complexes were visualized using an enhanced chemiluminescent detection system (West-Zol, Seoul, Korea) by incubating membranes with goat anti-rabbit

IgG or goat anti-mouse IgG antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), diluted at 1:2000. The blots were stripped and reprobed with an anti-β-actin polyclonal antibody to ensure that equal amounts of protein were used.

Investigation of Cytokine (IL-6, TNF-α) production by real-time RT-PCR

After treatment with 0.75-20 μg/ml LPS for 2, 4, 6, and 8 h, total RNA was isolated from the cells using Trizol reagent (Life Technologies Inc, Gaithersburg, MD, USA) according to the manufacturer's suggested protocol, and treated with DNase I (RNasefree, RQ1; Promega, Madison, WI, USA). One microgram of total RNA was used as a template to create first-strand cDNA with oligo-dT priming using an Omniscript RT kit (Qiagen Inc., Valencia, CA, USA). The quantitative real-time RT-PCR analyses were performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a SYBR Premix Ex Taq II kit (Takara, Otsu, Japan) and 35 cycles of PCR. The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s, respectively. The relative amount or fold change of the target gene was normalized relative to the level of the control (untreated cells). The following primer sequences were used in the real-time RT-PCR reactions ; 5′-CGA AAG TCA ACT CCA TCT GCC- 3′ and 5′-GGC AAC TGG CTG GAA GTC TCT-3′ for IL-6 gene ; 5′-CCA GGA GAA AGT CAG CCT CCT-3′ and 5′-TCA TAC CAG GGC TTG

AGC TCA-3′ for TNF-α gene ; 5’-GTG GTG GAC CTG ACC TGC-3’ and 5’-TGA GCT TGA CAA AGT GGT CG-3’ for GAPDH gene

Antimicrobial susceptibility assay of GV1001 peptide against *P. gingivalis*

GV1001 peptide was dissolved in phosphate buffered saline (PBS; pH7.2) and incubated at 4°C for 12 h to form tertiary structure. Antimicrobial susceptibility test of GV1001 peptide for *P. gingivalis* ATCC 33277 was carried out according to the recommendations of the Clinical and Laboratory Standards Institute (21). The bacteria were cultured in exponential phase and harvested by centrifugation at 4,000 x g for 10 min at 4°C. *P. gingivalis* were washed three times and resuspended with PBS. The number of bacteria was counted with Petroff-Hasser counting chamber (Hausser Scientific, Horsham, PA, USA) and inoculated into 96-well polystyrene plates (SPL Lifescience, Pocheon, Korea) containing 10 μl of PBS with or without various concentration of the peptide over a serial two-fold dilution range in PBS. The plates including the bacteria was incubated for 1 h at 37°C in anaerobic condition, and the oxygen-removed BHI broth including vitamin K and hemin was added into each well. The bacteria were incubated for 3 days at 37°C in anaerobic chamber. Growth of the bacteria was measured with spectrophotometer at 660 nm.

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD) of at least 3 separate experiments. Comparisons between 2 groups were analyzed using Mann-whitney U test. All analyses were performed by SAS (version 8.02, SAS Institue, Cary, NC, USA). *P*-values less than 0.05 were considered statistically significant.

3. Results

Verification of extracted LPS from *P. gingivalis*

The extracted LPS from *P. gingivalis* was analyzed by gel staining after SDS-PAGE. In case of staining with coomassie blue, the gel did not show any band. However, staining with silver nitrate exhibited the ladder pattern (Fig. 1). Also, staining the agarose gel with ethidium bromide did not show any band. These results indicated that the extracted LPS from *P. gingivalis* was uncontaminated with bacterial proteins or nucleic acids.

The receptors of extracted LPS from *P. gingivalis* (1 μg/ml), *S. aureus* LTA (1 μg/ml) and *E. coli* LPS (100 ng/ml) were analyzed by CD25 expression in CHO/CD14/TLR2 and CHO/CD14/TLR4 cell lines. CD25 was expressed in CHO/CD14/TLR2 cells by *P. gingivalis* LPS, *S. aureus* LTA and *E. coli* LPS with cell distribution of 27.54%, 28.92% and 45.87%, respectively. However, CHO/CD14/TLR4 cells were not affected by *P. gingivalis* LPS and *S. aureus* LTA but CD25 expression was induced by *E. coli* LPS (Fig. 2). These results indicated that *P. gingivalis* LPS and *S. aureus* LTA are agonists of TLR2, and *E. coli* LPS stimulates both TLR2 and TLR4. For the analysis of the cell distribution, non-treated cells as the control group was set 4.92%.

Internalization of GV1001 peptide

As shown in Fig. 3, GV1001 peptides (1, 10 and 50 μM) labeled with FITC at the C-terminus (FITC-GV) were observed in the cytoplasm of hDPC which confirms cell

penetrating ability of the peptide.

Cytotoxicity of GV1001 peptide in hDPCs

hDPCs were treated with GV1001 peptide, and cytotoxicity was tested by MTT assay. GV1001 peptide did not affect the viability of hDPCs in all concentration tested (1-50 μM), which showed a pattern similar to that of 0.9% NaCl ($P < 0.05$) (Fig. 4).

Inflammatory cytokine expression by *P. gingivalis* LPS

To examine the effects of extracted LPS on the induction of inflammatory cytokine expression in hDPCs, the levels of TNF-α and IL-6 expression were evaluated by real-time RT-PCR after exposing hDPCs to 1 μg/mL of *E. coli* LPS and *P. gingivalis* LPS at the concentrations of 1, 5, 10, and 20 μg/mL for 10 h. The results showed that IL-6 was profoundly induced in the presence of 1, 5, 10, and 20 μg/mL of *P. gingivalis* LPS and 1 μg/mL of *E. coli* LPS ($P < 0.05$), whereas TNF-α was significantly induced in the presence of 10 and 20 μg/mL of *P. gingivalis* and 1 μg/mL of *E. coli* LPS ($P < 0.05$) (Fig. 5A).

To evaluate the effects of LPS on inflammatory cytokine expression in hDPCs over a period of time, the levels of TNF-α and IL-6 expression were evaluated by real-time RT-PCR after exposing hDPCs to 20 μg/mL *of P. gingivalis* LPS or 1 μg/mL of *E. coli* LPS for 2, 4, 6, or 8 hrs. Up-regulation was found in hDPCs after 4 to 8 h of exposure to 20 μg/mL of *P. gingivalis* LPS and 1 μg/mL of *E. coli* LPS ($P < 0.05$) (Fig. 5B). The

expression of TNF-α and IL-6 in hDPCs were upregulated by the LPS in a time- and dose-dependent manner.

Inhibitory effect of GV1001 peptide on induction of TNF-α and IL-6 by *P. gingivalis* LPS

The levels of TNF-α and IL-6 were measured after LPS stimulation to investigate the anti-inflammatory activity of GV1001 peptide by real-time RT-PCR. LPS-stimulated TNF-α and IL-6 production was significantly inhibited ($P < 0.05$) (Fig. 6A and 7A).

Decrease of activated ERK and p38 MAPK by GV1001 peptide

To investigate whether the anti-inflammatory effect of GV1001 peptide was mediated by inhibition of LPS binding on TLR2 or by inhibition of the signaling pathway, signaling molecules related with TLR2 were investigated by western blot analysis. As shown in Fig. 6B and 7B, GV1001 peptide reduced LPS-induced phosphorylation of ERK and p38 MAPK.

Antimicrobial susceptibility test

As shown in Fig. 8, *P. gingivalis* showed antimicrobial susceptibility to GV1001 peptide at the concentration as low as 12 μM ($P < 0.05$) as compared to saline control.

4. Discussion

Although endodontically treated teeth can maintain their function for a prolonged period of time, there are many advantages to maintaining pulp vitality. In immature permanent teeth with incomplete apical and dentinal wall development, reparative dentin formation is critical for further development of the teeth. Maintaining the vital pulp also has the benefit of reducing the occurrence of apical periodontitis by blocking bacterial infections (22). Based on these advantages, it is important to maintain or renew pulp vitality. Recently, successful pulp regeneration and revascularization techniques have been developed and are gaining popularity.

P. gingivalis is an obligate anaerobic, gram-negative bacterium and is one of the most pathogenic species among black-pigmented gram-negative anaerobes (23); which is frequently present in root canal infections and odontogenic abscesses (24). *P. gingivalis* may also play a role in symptomatic infections, such as acute apical abscess (25, 26). Furthermore, there is a positive association between *P. gingivalis* and pain, mechanical allodynia, swelling and purulent exudates in root canals (26, 27).

Pathogens are generally recognized by specific receptors, one of which is the TLR family. TLR stimulation initiates host defense mechanisms through the activation of several intracellular signaling pathways, including activation of the MAP kinases and NF-κB proteins (28). Exposure of cells to inflammatory stimuli, including LPS and

proinflammatory cytokines, results in phosphorylation of MAPK (9).

Once the TLR is stimulated by a pathogen-associtated molecular pattern, proinflammatory cytokines and chemokines are produced by the odontoblast, resulting in recruitment and stimulation of immune effector cells and also direct bacterial killing (29). Although these biological responses protect the host against invading pathogens, the inflammatory response also leads to host tissue damage. Hence, regulating the production of these cytokines is pivotal to protecting host tissue.

The extra-telomeric functions of hTERT are suggested regarding cellular proliferation, stem cell mobilization, anti-apoptotic, anti-aging, and antioxidant effects through mitochondrial stabilization, transcriptional regulation (15, 16). If the therapeutic use of hTERT could be developed, it could be used in the treatment of pulpal diseases. In this study, the potential use of a GV1001 peptide of human origin as a therapeutic agent to control pulpal inflammation was suggested.

The anti-inflammatory effect of GV1001 peptide was achieved by modulating the suppression of the activation of ERK and p38 MAPK and the subsequent cytokine production induced by *P. gingivalis* LPS stimulation. *P. gingivalis* and *E. coli* LPS induced TNF-α and IL-6 expression in hDPC in a time- and dose-dependent manner, while GV1001 peptide down-regulated IL-6 and TNF-α. hDPCs expressed TLR2 and up-regulated phosphorylated ERK and p38 MAPK in response to stimulation by LPS. From the similar suppression patterns of the two, it could be assumed that the down regulation of inflammatory IL-6 and TNF-α by GV1001 was dependent on p38 MAPK and ERK-

signaling respectively, thus indicating that MAPK signaling was associated with down-regulation of inflammatory cytokines in dental pulp cells by GV1001 peptide. As shown in Fig. 6, low concentration of GV1001 peptide was more efficient in lowering TNF-α induced by *P. gingivalis* LPS compared to the high concentration. One of the possible reason is that GV1001 peptide can partially aggregate in the high concentration since the cell culture medium does not contain carrier proteins. Thus, GV1001 peptide in low concentrations which did not aggregate, penetrate into the cells, and react with signaling molecules related to inflammation.

In another previous study, GV1001 peptide was reported to have the ability to penetrate into various cells, including cancer cell lines and primary blood cells, without affecting cell viability (14). GV1001 peptide was predominantly located in the cytoplasm and was used to successfully deliver macromolecules such as proteins, DNA and siRNA into cells (14). These cell-penetrating peptides (CPPs) have become one of the most popular and efficient tools for delivering various molecules into the cells owing to the fact that they have the ability to enter cells independently of a membrane receptor, and they show no cell-type specificity (30). In this study, GV1001 peptide was observed in the cytoplasm of hDPCs. This study is significant in that it is the first to demonstrate GV1001 peptide as a CPP of human origin to express anti-inflammatory effect of its own, without affecting cell viability in hDPCs.

GV1001 peptide had antimicrobial activity against *P. gingivalis* at the concentration as low as 12 μM and also had the ability to neutralize LPS toxicity by inhibiting

activation of signaling molecules thus providing a possibility of applications for antimicrobial agent.

Furthermore, future application for vital pulp therapy can be considered; such as the use of GV1001 peptide as a pulp-capping agent on reversibly inflamed pulp or an alternative to antibiotics in regeneration therapy. The peptide can also be used as an intracellular delivery tool for bioactive molecules as shown in the previous study (14). Conjugating growth factors such as TGF-βs and BMPs with GV1001 peptide may facilitate induction of hDPCs to differentiate effectively into odontoblast-like cells. Moreover, further studies may provide an insight for gene therapy to fuse a growth/differentiation factor with GV1001 peptide for application in tissue engineering. The results from this study may support further research on GV1001 peptide and its various clinical applications.

5. Conclusions

GV1001 peptide had the ability to penetrate into the cell. The peptide downregulated *P. gingivalis* LPS-induced TNF-α and IL-6 expression through the inactivation of ERK and p38 MAP kinase pathways. These findings provide the insight of how GV1001 peptide causes anti-inflammatory activities in LPS-stimulated pulpal inflammation without significantly affecting host cell viability. GV1001 peptide also showed a potential of antimicrobial property against *P. gingivalis*. Collectively, GV1001 peptide may have preventive and therapeutic effect on infectious pulpal inflammation induced by *P. gingivalis*.

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Figures

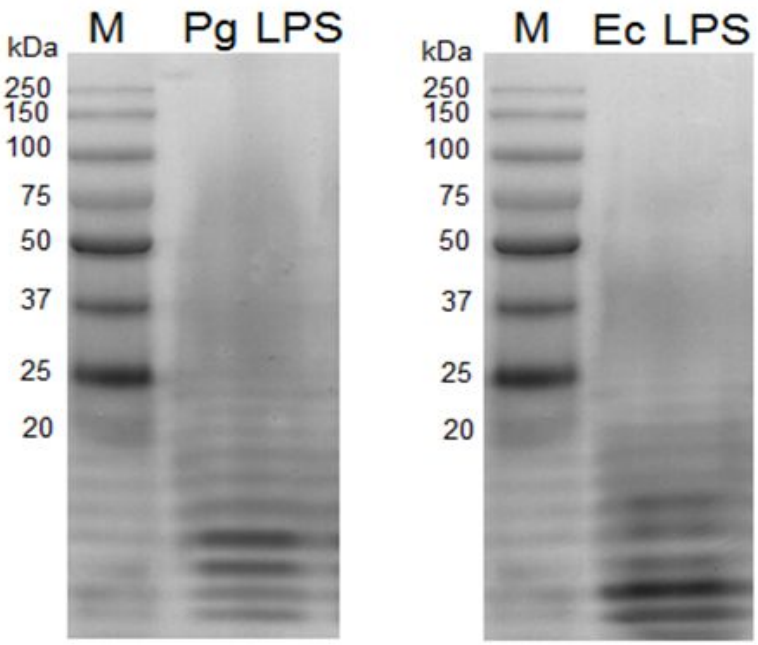

Figure 1. SDS-PAGE of *P. gingivalis* LPS. The LPS was isolated from *P. gingivalis* by LPS extraction kit. SDS-PAGE (10%, acrylamine) was performed, and the gel was stained with silver nitrate. *E. coil* LPS was used as a standard molecule. Pg LPS: *P. gingivalis* LPS, Ec LPS: *E. coli* LPS, and M; size marker.

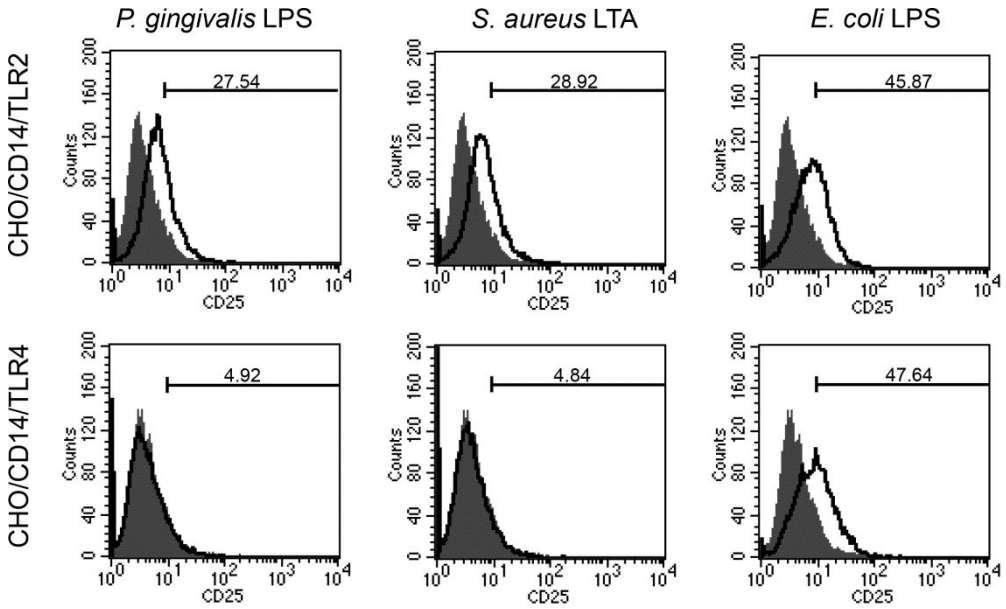

Figure 2. Biological activity of *P. gingivalis* LPS. CHO/CD14/TLR2 and CHO/CD14/TLR4 cells were evaluated to confirm which TLR was used by *P. gingivalis* LPS. CD25 expression in the cells was analyzed by flow cytometry, and the data and expressed gate % were analyzed after setting 4.92% of control group.

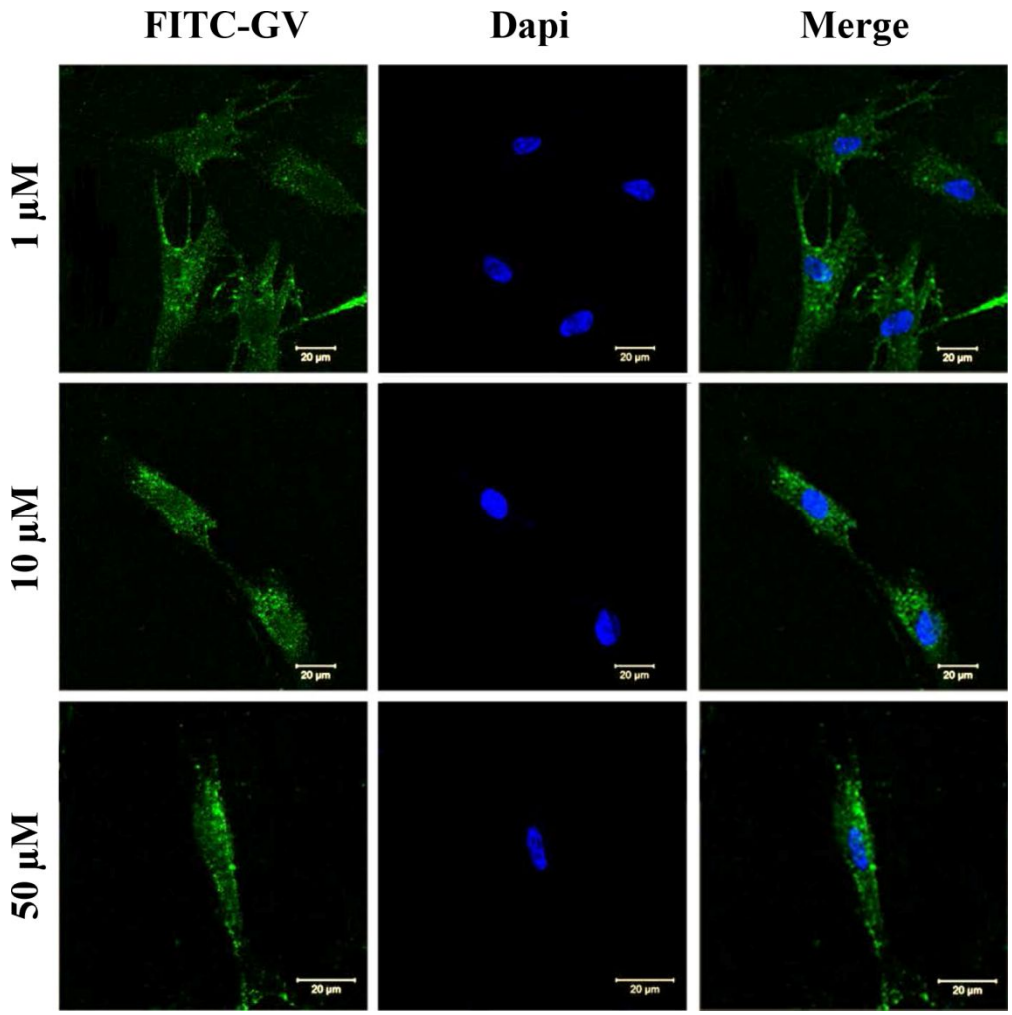

Figure 3. Internalization of GV1001 peptide into hDPCs. 1, 10 and 50 μM of GV1001 peptides labeled with FITC at the C-terminus (FITC-GV) were used to treat hDPCs as described in the Materials and Methods. Internalization of the peptides was analyzed by confocal microscopy.

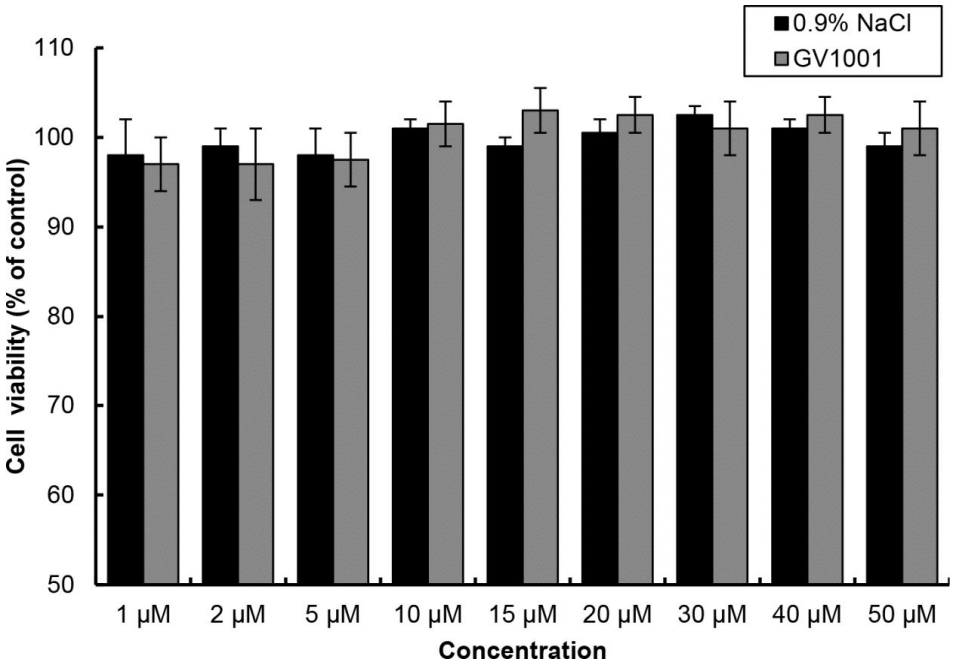

Figure 4. Effects of GV1001 peptide on viability of hDPSCs. The cells (2×10^5 cells/well) were treated with the indicated concentrations of GV1001 peptide for 48 h. The cell viability was assessed by MTT assay and the surviving cell values were shown as the percentage of the control-treated cells (no addition of GV1001 peptide); 0.9% NaCl solution was used as a negative control. Each value indicates the mean ± SD of three independent experiments.

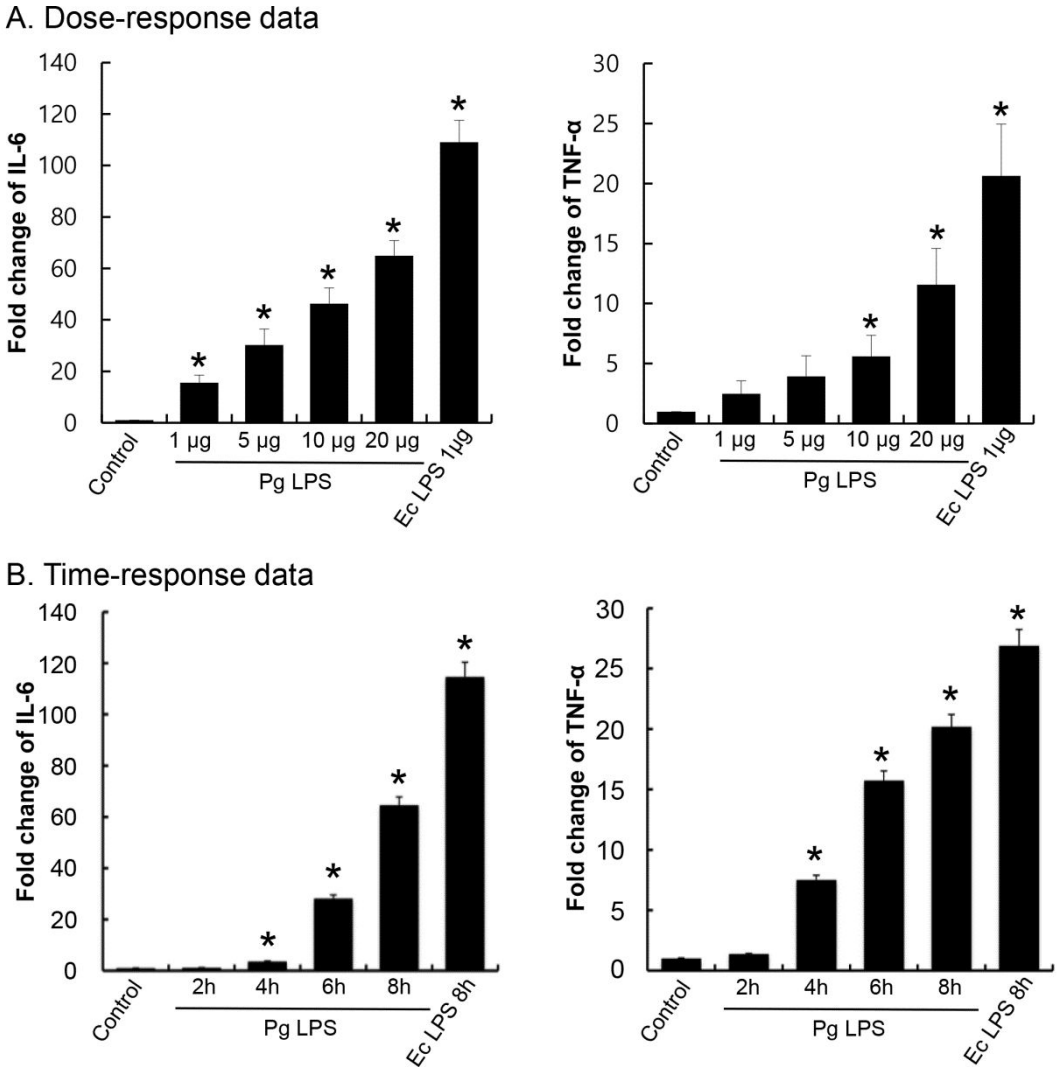

Figure 5. Effects of various doses of LPS and exposure times on the expression of IL-6 and TNF-α in hDPCs. hDPCs were serum-starved for 24 h and treated with indicated concentrations of *P. gingivalis* LPS (1, 5, 10, and 20 μg/mL) (A), for different times (2, 4, 6, and 8 h) (B). The levels of IL-6 and TNF-α mRNAs were determined by real-time RT-PCR. Each value indicates the mean ± SD of three independent experiments.

* indicates a significant difference ($P < 0.05$) relative to non-treated cells as control. 1 μg/mL of *E.coli* LPS was treated as a positive control.

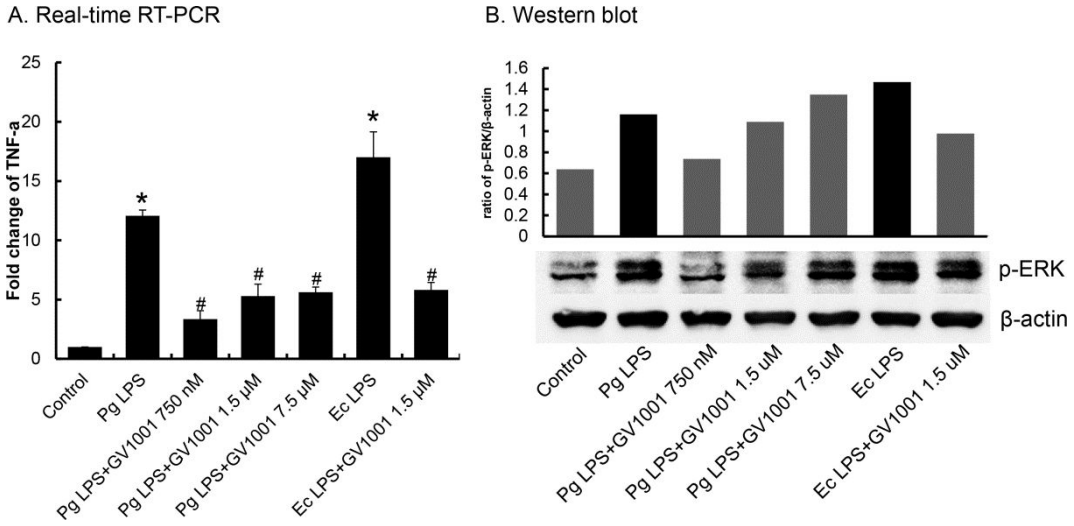

Figure 6. Effects of GV1001 peptide on LPS-induced TNF-α and p-ERK production in hDPCs. 0.75, 1.5 and 7.5 μM of GV1001 peptide was added to hDPSCs treated with 1 μg/mL of *E. coli* LPS or 20 μg/mL of *P. gingivalis* LPS for 10 h. Real- time RT-PCR and western blot revealed down-regulation of (A) TNF-α and (B) phosphorylated ERK, respectively, after exposure of cells incubated with GV1001 peptide and 1 μg/mL of *E. coli* LPS or 20 μg/mL of *P. gingivalis* LPS. Each value indicates the mean ± SD of three independent experiments.

* indicates a significant difference ($P < 0.05$) relative to non-treated cells as control.

# indicates a significant difference ($P < 0.05$) relative to cells treated with LPS in the presence of GV1001 peptide.

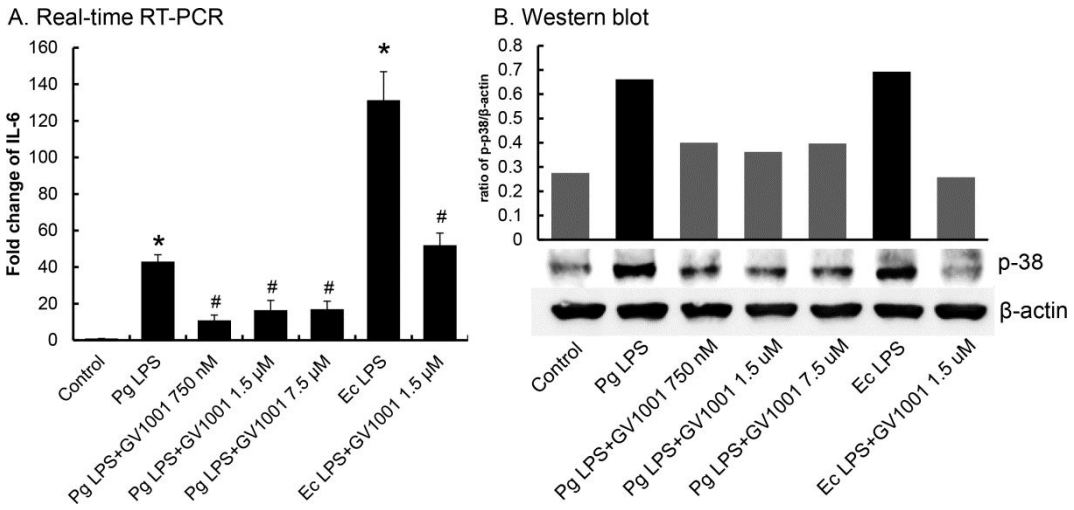

Figure 7. Effects of GV1001 peptide on LPS-induced IL-6 and p-p38 expression in hDPCs.

0.75, 1.5 and 7.5 μM of GV1001 peptide was added to hDPCs treated with 1 μg/mL of *E. coli* LPS or 20 μg/mL of *P. gingivalis* LPS for 10 h. Real-time RT-PCR and western blot revealed down-regulation of (A) IL-6 and (B) phosphorylated p38, respectively, after exposure to 1 μg/mL of *E. coli* LPS or 20 μg/mL of P. *gingivalis* LPS incubated with GV1001 peptide (0.75, 1.5 μM and 7.5 μM). Each value indicates the mean ± SD of three independent experiments.

* indicates a significant difference ($P < 0.05$) relative to non-treated cells as control.

# indicates a significant difference ($P < 0.05$) relative to cells treated with LPS in the presence of GV1001 peptide.

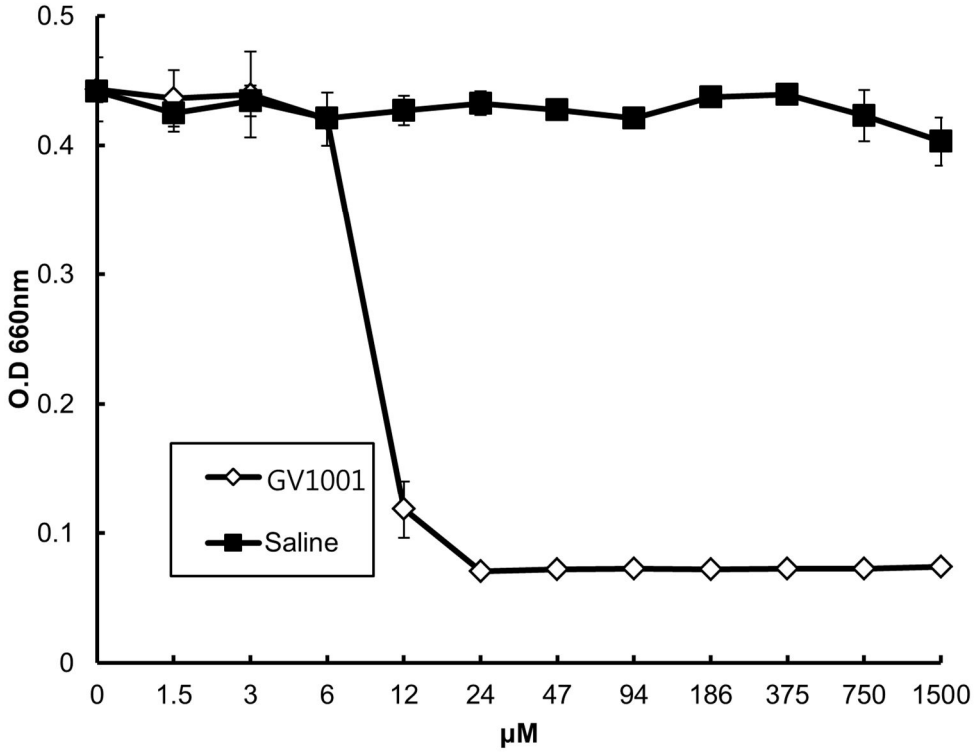

Figure 8. Antimicrobial susceptibility test of GV1001 peptide for *P. gingivalis* was carried out according to the recommendations of the Clinical and Laboratory Standards Institute. Growth of the bacteria was measured with spectrophotometer at 660 nm.

국문초록

사람 Telomerase 유래 펩타이드가 *Porphyromonas gingivalis* Lipopolysaccharide 에 의해 유도된 사이토카인 발현에 미치는 항염증 효과에 관한 연구

고 유 진

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1. 목적

치수 및 치근단 질환의 주된 원인인 세균감염을 억제하고 조절하기 위해 항균, 항염증 펩타이드가 우식 및 근관치료 영역에 소개되고 있다. Human telomerase reverse transcriptase (hTERT) 유래의 항암 백신으로 개발된 GV1001 펩타이드는 세포내로 들어가 약리학적 효과를 나타내는 것과 함께, 항염 효과도 보고되고 있어, 치수 염증 질환 치료에의 적용 가능성을 제시하

였다. 본 연구의 목적은 사람 치수 세포에서 *P. gingivalis*의 LPS로 유도된 염증에 대한 GV1001 펩타이드의 항염 효과를 알아보기 위함이다.

2. 재료 및 방법

성인의 매복 제3대구치에서 치수세포를 분리 배양하였다. *P. gingivialis* LPS (1 μg/ml)가 어떠한 Toll-like receptor (TLR)를 자극하는지 확인하기 위하여 CHO/CD14/TLR2와 CHO/CD14/TLR4를 사용하여 검증하였고 *S. aureus* LTA (1 μg/ml)와 *E. coli* LPS (100 ng/ml)는 대조군으로 사용하였다. Confocal microscopy로 GV1001 펩타이드의 세포질내 투과를 확인하였다. 사람 치수세포에 대한 GV1001 펩타이드 (1-50 μM)의 세포독성은 MTT assay로 분석하였고, *P. gingivalis*의 LPS로 유발된 염증 사이토카인인 TNF-α와 IL-6의 발현양을 real-time RT-PCR로 분석하였다. GV1001 펩타이드의 항염 효과에 MAP kinase (ERK, p38) 신호전달 과정이 관여하는지를 western blot 분석으로 확인하였다. 만-휘트니 U검증을 통하여 통계학적 유의성을 검증하였다 (P=0.05).

3. 결과

P. gingivalis LPS는 TLR2를 통해 CD25를 발현하였다. GV1001 펩타이드가 사람 치수 세포내로 들어가 세포질 내에 분포하였고, 사람 치수 세포에서 세포독성을 나타내지 않고 *P. gingivalis* LPS로 유도된 TNF-α와 IL-6의 생성

을 유의하게 감소시켰다 ($P < 0.05$). 또한 GV1001 펩타이드는 LPS로 자극된 사람 치수 세포에서 MAP kinases (ERK, p38)의 인산화를 현저하게 억제하였다.

4. 결론

GV1001 펩타이드는 사람 치수 세포에서 *P. gingivalis* LPS로 유도된 ERK와 p38 MAPK의 인산화 억제를 통해 TNF-α와 IL-6의 발현을 감소시켜 항염 효과를 나타내었다.

주요어: GV1001 펩타이드, 사람 telomerase 유래 펩타이드, *Porphyromonas gingivalis* LPS, MAP kinase (ERK, p38), 항염 효과
학 번: 2012-30589